A 23 kDa CHLOROPLAST PROTEIN AS MARKER FOR DROUGHT TOLERANCE IN *HEVEA BRASILIENSIS*

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Climate-resilient smart rubber clones need to be developed for expansion of rubber cultivation to regions where environmental stresses are a limiting factor. In this context physiological responses of young plants belonging to ten clones of *Hevea brasiliensis* were analyzed under soil moisture deficit condition. The relative drought tolerance potential of these clones were evaluated using key physiological parameters such as leaf water potential, photosyntic oxygen evolution rate of leaf, quantum yield of PS II and CO_2 assimilation rates. Clones *viz*. RRIM 600, RRII 208 and RRII 430 were more drought tolerant even as they recorded comparatively less decline in photosynthesis and PS II activity. On the other hand, clones PB 260, RRII 105, RRII 414 and RRII 417 were relatively more drought susceptible in terms of severe inhibition of various photosynthetic activities under moisture stress. Western blot analysis of a low molecular weight (23.8 kDa) chloroplast stress protein indicated its consistent over-expression in the relatively drought tolerant clones when these plants were subjected to water deficit stress. Abundance of this protein was associted with relatively lesser inhibitions in photosynthetic activity in drought stressed young rubber plants. The relative expression level of this protein together with other crucial physiological parameters such as photosynthetic activity can be used as potential screening tools for selection of drought tolerant clones at a young stage.

Key words: Abiotic stress, Chloroplast stress protein, Drought tolerance, *Hevea brasiliensis*, Physiological marker, Screening tool

INTRODUCTION

To meet the increasing global demand for natural rubber and considering its limited scope of expansion in the traditional belts, attempts are being made to extend its cultivation to marginally suitable areas in several countries with varied climatic constraints. In India, cultivation of rubber is being extended to non-traditional areas in the Konkan region, eastern ghat areas and North East India. Adverse environmental conditions such as drought, high and low temperatures, high light and high vapour pressure deficit (VPD), poor soils etc., limit the expansion of rubber cultivation to newer areas in several rubber producing countries including India (Sethuraj *et al.*, 1989; Samarappuli and Yogaratnam, 1998; Jacob *et al.*, 1999). Climate change as a result of global warming can influence the growth and productivity of natural rubber (Satheesh and Jacob, 2011). Abiotic stresses affect every aspect of plant growth, anatomy, physiology, biochemistry and gene expression. Drought

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is probably the single most important largest factor which restricts the expansion of cultivation of *Hevea brasiliensis* to newer areas. Soil and atmospheric drought combined with high solar light intensity have been reported as major environmental constraints for establishing rubber plantations in areas such as the North Konkan (Devakumar *et al.*, 1998; Jacob *et al.*, 1999; Alam *et al.*, 2005).

In recent years, it is observed that climate is becoming a limiting factor in the traditional rubber growing areas too. Countries such as India, Thailand and Sri Lanka are experiencing drought stress in the traditional belts and this can be a major constraint in the early stage of establishment of rubber plantations (Samarappuli and Yogaratnam 1998; Chantuma *et al.*, 2012; Jessy *et al.*, 2014). Though, rubber can be grown successfully in non-traditional regions with adequate irrigation during summer period (Vijayakumar *et al.*, 1998) availability of irrigation water and labor are challenging problems in most of the rubber growing countries. Most of the field grown plants tolerate environmental stresses through many metabolic adaptations at cellular and organelle level such as effective reactive oxygen and free radical scavenging systems, photo-protection of pigment-protein complexes through excess electron dissipation mechanism (Demmig-Adams and Adams, 2006) and expression of specific stress proteins (Vierling, 1991). A consistently over - expressing chloroplast stress protein was reported and implicated in drought response in young plants of *Hevea* (Annamalainathan *et al.*, 2006). However, the pattern of expression of this chloroplast protein was not validated with functional aspects of photosynthetic apparatus and drought tolerance potential of different clones. Breeding and developing new rubber clones with increased tolerance to drought condition is essential, especially in the present scenario of global warming and climate change. In this context, attempts need to be made to identify critical physiological and biochemical traits which characterize the *Hevea* genotypes for drought tolerance. Establishing specific markers are essential for screening vast number of elite hybrid *Hevea* clones and other ortets and hybrids in the pipeline for stress tolerance.

In the present study, drought response of ten *Hevea* clones were assessed by measuring certain crucial physiological activities like photosynthetic oxygen evolution rate, effective quantum yield of PS II and net CO_2 assimilation rate (P_N). Abundance of a 23 kDa stress responsive protein (Annamalinathan *et al.*, 2006) in chloroplast was quantified and related with photosynthetic activities of the ten different clones under drought. The feasibility of using the relative expression level of this stress protein to screen rubber clones for drought stress tolerance is discussed.

MATERIALS AND METHODS

Plant material and growth condition

Budded stumps of ten clones of *Hevea, viz*. RRII 105, RRII 208, PB 260, RRIM 600, RRII 414, RRII 417, RRII 422, RRII 429, RRII 430 and Tjir 1 were planted in large (35 x 65 cm) size polythene bags filled with 30 kg of soil. The plants were grown under normal field conditions (twenty plants per treatment) with open sunlight for one year. One set of plants of each clone was drought stressed by withholding irrigation for 10 days during the rain free summer season. Mid-day sun light load was around 1800 µmol $m^{-2}s^{-1}$. A second set of plants was kept as irrigated control. Photosynthetic measurements were made in these plants followed by leaf sample collection for chloroplast protein analysis.

Measurement of leaf water potential (Ψ_L)

The mid-day (12:00 noon) water potential of the leaf from sun exposed top whorl of irrigated and drought imposed plants was measured using Psypro water potential system(Wescor). Psychrometer measures the water vapor pressure of a solution or sample on the basis of the principle that evaporation of water from a surface cools the surface. The sample chambers of Wescor system were taken to the field and leaf discs collected were immediately transferred to the chambers, transported to laboratory and then observations were taken.

Measurements of photosynthetic oxygen evolution

The rate of photosynthetic oxygen evolution by leaf discs of freshly harvested leaf (with an area of 9.2 cm^2) was measured at 25^0C with a Clark type oxygen electrode (Hansatech LD 2/2, King's Lynn, UK). The measurement light (LED) was produced using a Hansatech LH 36 light source. To avoid any CO_2 limitation, two per cent CO_2 was generated in the closed chamber using 100mM bicarbonate buffer (pH 9.2). The leaf disc was first acclimatized to complete darkness for five minutes to achieve full potential dark respiration. The leaf disc was then exposed to light intensity (500 µmol $m^{-2}s^{-1}$) using a LED source for five minutes and photosynthetic oxygen evolution rate was measured (Walker, 1988).

Measurement of photosynthetic CO_2 assimilation

An infra-red gas analyzer (IRGA), Li 6400 (LiCOR, Nebraska, USA) was used to measure the net photosynthetic rate (P_N) of the plants. The required measurement light intensity (500 µmol $m^{-2}s^{-1}$ red light + 10% blue light), CO_2 concentrations (400ppm) and other environmental parameters like temperature and RH were maintained with normal ambient conditions (Sumesh *et al.*, 2011).

Assay of quantum yield of PS II

The chlorophyll fluorescence technique is a simple, non-destructive method widely used to assess the physiological state of photosynthetic apparatus during any environmental stress condition (Krause and Weis, 1991). Chlorophyll fluorescence was measured at a steady state intensity of 316µmol $m^{-2}s^{-1}$ actinic light by using PAM 2100 (Waltz, Germany) and PS II quantum yiels ($\Phi_{PS\ II}$) was calculated (Schreiber *et al.*, 1998).

Isolation of chloroplasts

Type II broken chloroplasts were isolated by the method of Fish and Jagendorf (1982). Fresh leaf sample was ground with liquid nitrogen using mortar and pestle. The powdered leaf sample was added with 5 ml of grinding buffer (Tris buffer pH 7.8) and transferred to a centrifuge tube. The filtered homogenate was centrifuged at 800g for two minutes. The pellet representing unbroken cells and tissue was removed and the supernatant was spun at 3500g for five minutes. The resulting pellet was suspended in 1 ml of Tris buffer (pH 7.8) as chloroplast suspension. The chloroplasts were further subjected to 80 per cent acetone and 10 per cent TCA wash in order to remove pigments and lipids and proteins were pelleted out.

Western blot analysis of a 23kDa stress protein

Chloroplast proteins were loaded into a polyacrylamide gel containing a denaturing agent (SDS) and the proteins were separated in the gel (Laemmli, 1970). Chloroplast protein profile was transferred from the gel onto a nitrocellulose or PVDF membrane

following Towbin's (1979) method of transfer which was then incubated with a polyclonal primary antibody directed against the 23 kDa protein which was earlier detected in a few drought tolerant clones (Annamalainathan *et al.*, 2006). The unbound antibody was washed off using TBS buffer (Tris-buffered saline) leaving only the bound antibody to the protein of interest. The bound antibodies were then detected by developing the membranes. By using a detector conjugated to a secondary antibody the protein of interest was specifically detected. The magnitude of expression was quantified from the relative band intensity using a Gene Genius Bio-imaging system, Syngene, USA. For consistent values, the blotting experiment was repeated thrice.

Statistical analysis of data

Two factor ANOVA was performed to test the difference between clones for various physiological activities. Comparisons were made between clones and drought effect interactions using Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Physiological response of young rubber plants to drought stress

The mid-day Ψ_L among irrigated plants did not show any significant clonal differences except in RRII 414 (Fig. 1). However, Ψ_L showed significant differences between the irrigated and drought exposed plants in all the clones. Drought imposed plants recorded significantly less leaf water potential than to their irrigated counterparts. There were significant clonal differences existing among the drought imposed plants (Fig. 1). Clones such as RRIM 600, RRII 429, RRII 430 and Tjir 1 maintained relatively better Ψ_L after 10 days of withdrawal of irrigation; whereas, clones, RRII 105, PB 260, RRII 414 and RRII 422 showed more reduction in leaf water potential indicating their fair degree of susceptibility to desiccation stress in these clones.

In general the effective quantum yield of PS II (Φ_{PSII}) declined in all the drought imposed plants when compared to their irrigated counterparts (Fig. 2). The magnitude of inhibition was severe in clones PB 260, RRII 105, RRII 414, RRII 417 and RRII 422. On the other hand clones RRIM 600 and RRII 430 showed relatively better stability in PS II activity after 10 days of drought imposition (Fig. 2). The extent of inhibition was moderate in RRII 429 and Tjir 1.

The most commonly used protocol for measuring the photosynthetic efficiency of photosystem II under a stress condition is effective quantum yield of PS II, a crucial indicator to analyze the potential of PS II in light exposed plants. It is an indication of the amount of energy utilized in photochemistry by PS II under steady-state photosynthetic lighting conditions. The present result demonstrates differential response of PS II activity to drought associated water deficit and high temperature stresses in young plants of different clones.

The photosynthetic oxygen evolution rate was measured in leaves of irrigated and drought imposed plants in the morning hours (9:00-10:00 am). The activity was significantly different among the irrigated plants of various clones (Fig. 3). The rate of activity was significantly lesser in PB 260, RRII 430 and Tjir 1 than other clones. Under drought condition, the activity was drastically inhibited compared to their respective irrigated control plants in clones RRII 105, PB 260, RRII 414 and Tjir1. But this was less inhibited in RRIM 600, RRII 430, RRII 208 and RRII 429 and they had similar level of O_2 evolution during stress (Fig. 3). It is known that the O_2

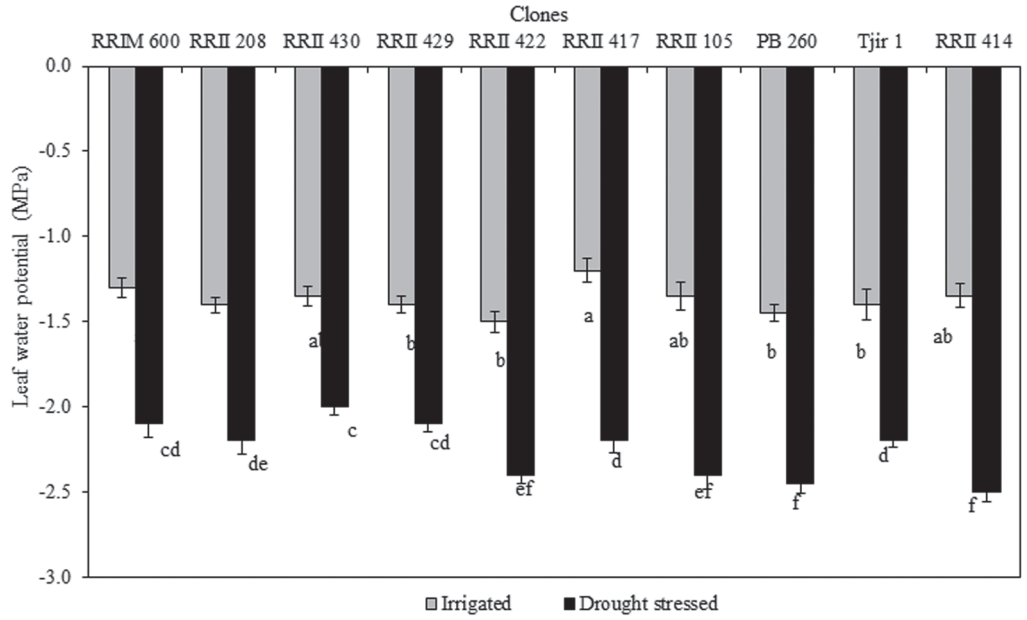

Fig. 1. Mid-day leaf water potential of young plants of different clones of Hevea grown in polybags. Irrigation was withheld for 10 days in drought samples. Control plants were irrigated continuously to saturated soil moisture level

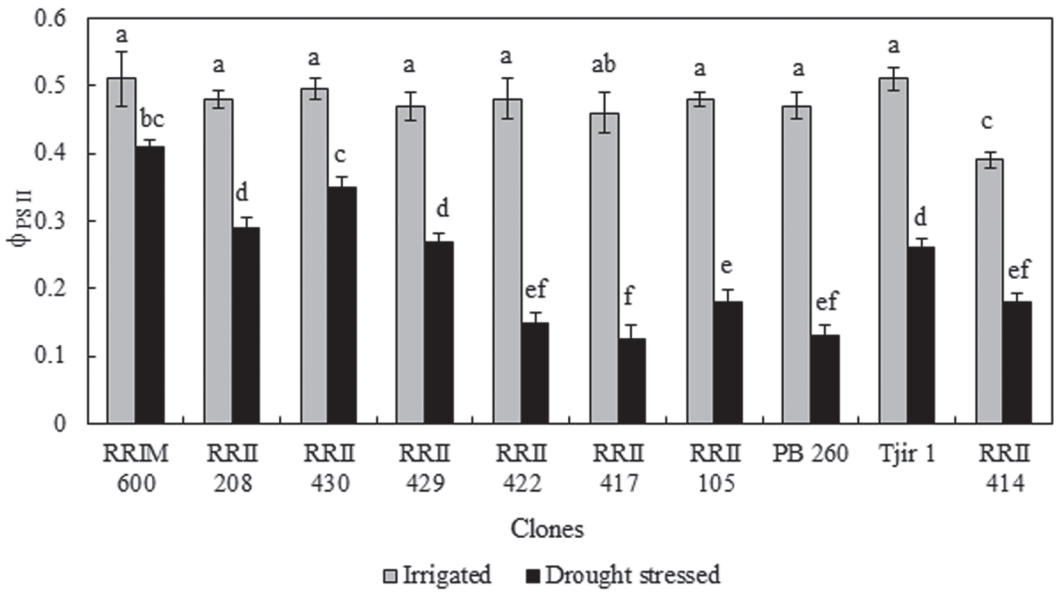

Fig. 2. The effective quantum yield of photosystem II ($\Phi_{PS\ II}$) in young rubber plants exposed to drought stress for 10 days by withholding irrigation in polybags. The control plants were maintained at saturated level of soil moisture

evolving complex (OEC) is the most susceptible component in the photosynthetic apparatus to water and high temperature stresses in many plants (Berry and Bjorkman, 1980).

The net photosynthetic CO_2 assimilation rate measured during morning hours (9:00-10:00 am) also showed a similar trend as observed for photosynthetic oxygen evolution activity (Fig. 4). However, clones such as RRII 417 and Tjir 1 recorded very low P_N after withdrawal of irrigation (Fig. 4). The drastic reduction in P_N indicated their relative susceptibility to desiccation stress. Although clones such as RRII 208, RRIM 600 and RRII 430 recorded a significantly lesser rate of P_N than their respective irrigated controls, they maintained comparatively stable level of photosynthesis than other clones after 10 days of drought. The drastic inhibition of photosynthetic CO_2 assimilation rate under water deficit condition also substantiated the results observed on reduced PS II quantum yield in the light-adapted leaves (Fig. 2) and such reductions may be a mechanism to down-regulate photosynthetic electron transport to match decreased CO_2 assimilation in soil water stressed plants.

In the present study ten elite rubber clones were observed to be varied in their relative drought tolerance potential during early growth period. The popular and high yielding modern clones *viz.* RRII 105, PB 260 and RRII 414 were found relatively drought susceptible as observed from severe decline of leaf water potential, photosynthetic oxygen evolution rate, PS II quantum yield and net photosynthetic CO_2 assimilation under moisture deficit condition. On the other hand, clones such as RRIM 600, RRII 430 and RRII 208 could tolerate soil moisture stress by maintaining relatively better and stable photosynthetic activities under similar level of stress condition (with holding irrigation for 10 days). Gas exchange and

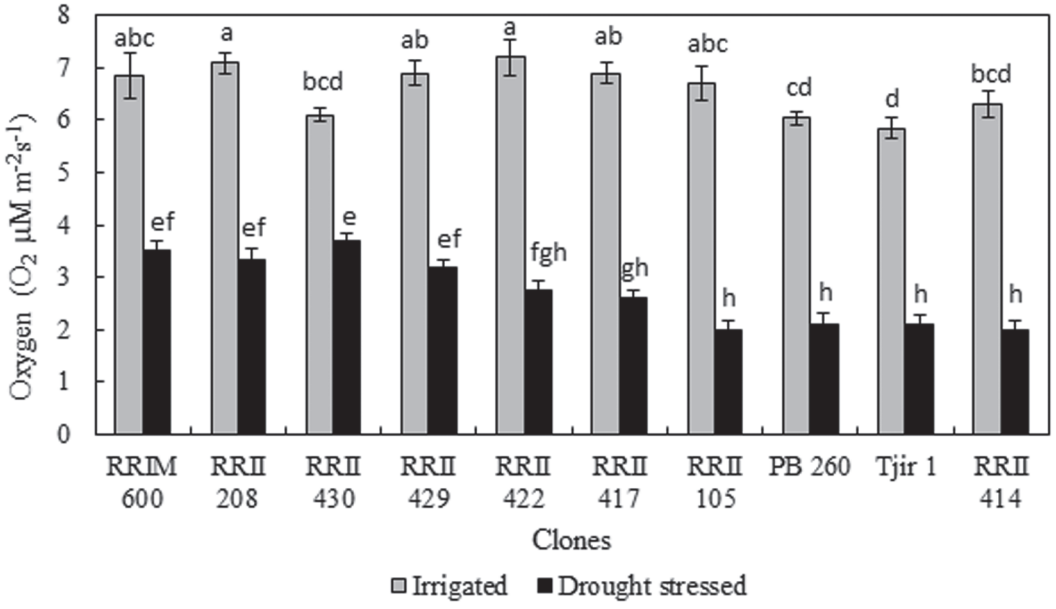

Fig. 3. Photosynthetic oxygen evolution rate of leaf discs collected from control (irrigated) and drought imposed (withholding irrigation for 10 days) plants of *Hevea*

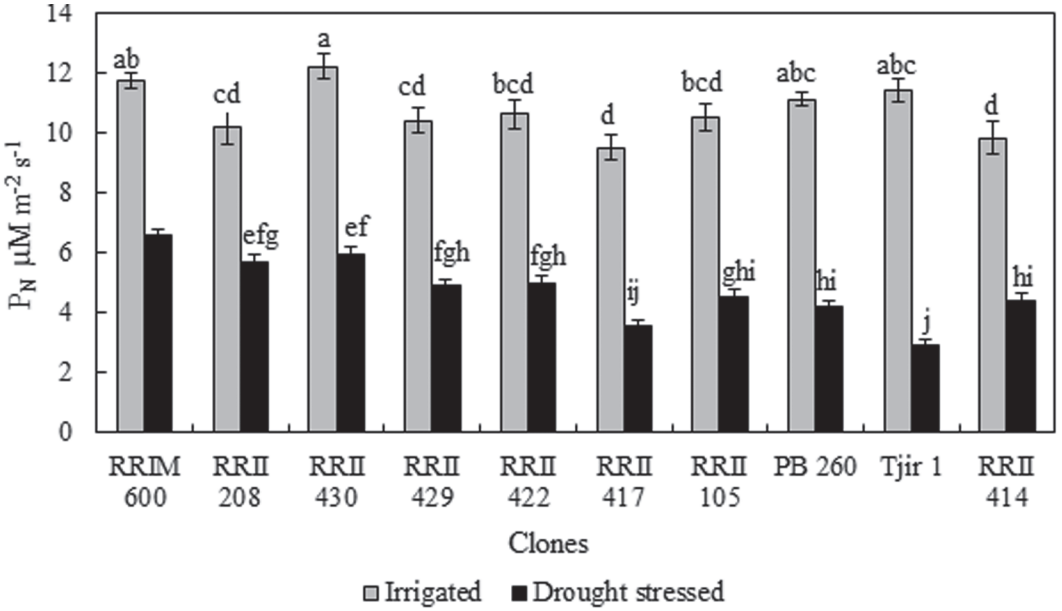

Fig. 4. Net photosynthetic rate (P_N) of *Hevea* plants exposed to drought stress for 10 days by withholding irrigation in polybags. The control plants were maintained at saturated level of soil moisture condition

fluorescence studies revealed that clone RRII 430 was more likely to endure drought stress better than the other clones including other RRII 400 series clones. Previous reports also show that RRIM 600 and RRII 430 were physiologically better adapted and can with stand water stress for a relatively longer period of time (Annamalainathan *et al.*, 2010; Sumesh *et al.*, 2011). The degree of susceptibility of RRII 105, a popular high yielding rubber clone in India, to drought condition has been well documented in many previous studies (Alam *et al.*, 2005; Annamalainathan *et al.*, 2006). Interestingly, one modern high yielding clone, RRII 414 is drought susceptible in terms of severe inhibition of various photosynthetic parameters under stressful growth conditions. The response of photosynthetic parameters to soil moisture deficit stress was genotype specific in modern *Hevea* clones. In general, most of the damaging effects of irradiation and moisture stress to green leaves occur at the chloroplast membrane and enzyme levels (Oquist *et al.*, 1995). The PS II and electron transport components of thylakoid membranes are the main targets of photoinhibiton due to the formation of excess active oxygen species during adverse climatic condition (Jacob and Karaba, 2000; Ashraf and Harris, 2013).

Chloroplast stress protein

A 23.8kDa chloroplast protein was observed to be over - expressing consistently in drought imposed young plants of *Hevea*. This protein was analyzed and identified by LC-MS/MS and mass spectrometry. Further non-redundant protein data base (MSDB) available through the Mascot search engine was used to match the polypeptides with several sHSPs of other reported species and this protein was reported as a hsp type protein associated with chloroplast thylakoid

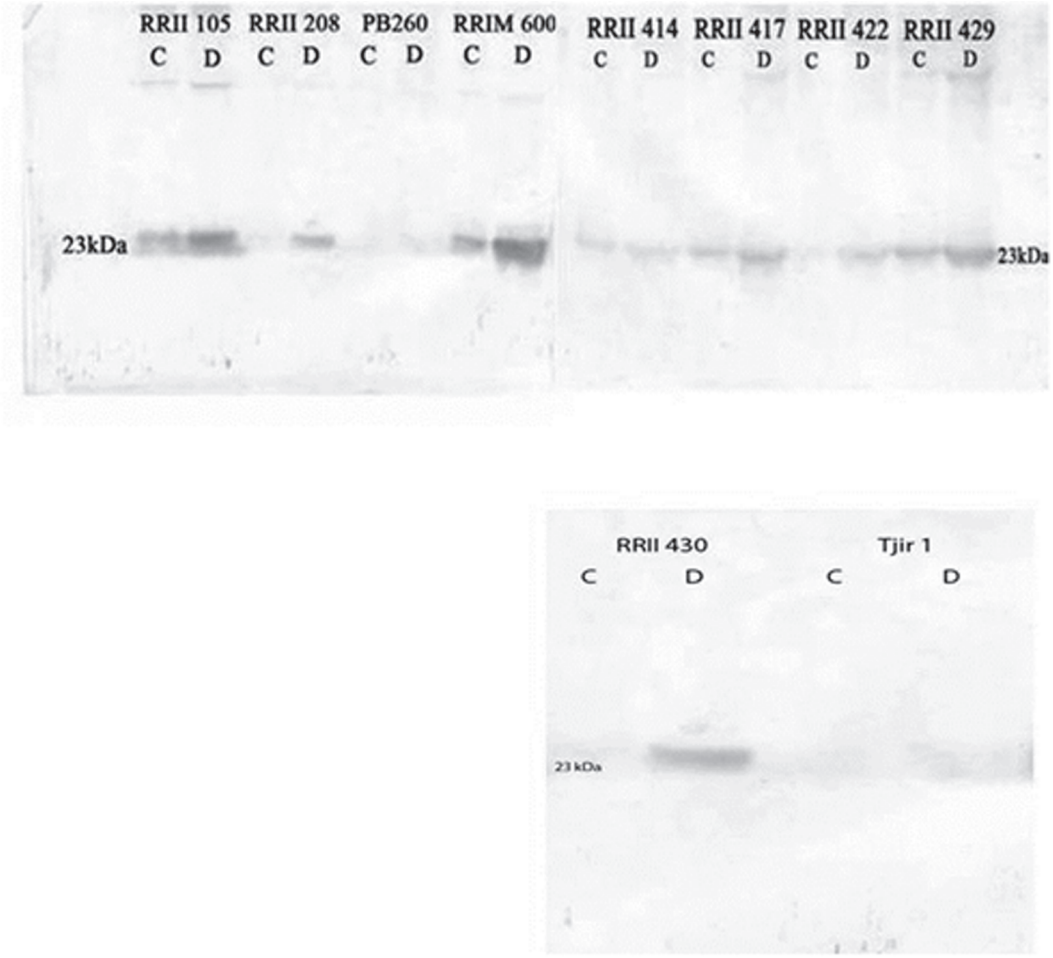

Fig 5. Western blot profile of 23.8 kDa chloroplast stress protein extracted from ten different Hevea clones (C-control; D-drought stressed). Chloroplast protein (50 µg) was loaded uniformly in each lane. The stress protein was detected by incubating the chloroplast protein profile in PVDF membrane with a polyclonal antibody raised against this protein

membrane in *Hevea* (Annamalainathan *et al.,* 2006). The antibody raised against this protein was used to detect and quantify the protein in Western blots. In control irrigated plants the accumulation of this protein was very minimum to obscure. On the contrary the water deficit stress imposed plants accumulated significantly high level of stress protein in the thylakoid membranes (Fig 5). The relative abundance of this hsp in the more drought tolerant clones (such as RRIM 600, RRII 430 and RRII 429) was very prominent than relatively drought susceptible clones (PB 260, RRII 414 and Tjir 1). The drought tolerant clones recorded around 71–110 per cent over-expression of this stress protein upon exposure to drought compared to their respective irrigated counterparts whereas the susceptible clones had a relative abundance of only 8- 30 per cent over their respective control plants (Table 1).

Interestingly, a well-known drought tolerant rubber clone RRIM 600 recorded the highest abundance (110 per cent) compared to the control irrigated plants. Those clones susceptible to water deficit stress in terms of large inhibition of PS II activity, photosynthetic oxygen evolution and net photosynthetic CO_2 assimilation rate under soil moisture deficit stress had very low level of this protein.

There was significant relationship between the abundance of this protein and relative drought tolerant traits among the clones studied (Fig. 6). The relatively more drought tolerance clones namely RRIM 600, RRII 430, RRII 429 and RRII 208 had respectively 110, 75, 72 and 71 per cent over-expression if this protein when exposed to drought stress. Therefore, the quantitative analysis of this protein along with measurement of crucial photosynthetic parameters like Φ_{PSII}, oxygen evolution and CO_2 assimilation rates could be used for the identification of relatively drought tolerant clones in young rubber plants. RRII 105, which is graded as drought susceptible in the present study, but had a relatively fair degree (around 30% over control plants) of accumulation of the 23 kDa stress protein under water deficit condition. The degree of drought susceptibility of this popular clone in India is some what debatable although it

Table 1. **The relative abundance of 23 kDa chloroplast stress protein in different clones of *Hevea* after exposure to 10 days of soil moisture deficit stress by withholding irrigation. The relative abundance was calculated keeping the value for respective control (irrigated) plants**

Clone	Increase of protein abundance in drought stressed samples over the respective irrigated control (%)
RRIM 600	110
RRII 430	75
RRII 429	72
RRII 208	71
RRII 105	30
RRII 417	28
RRII 422	18
Tjir 1	15
RRII 414	14
PB 260	8

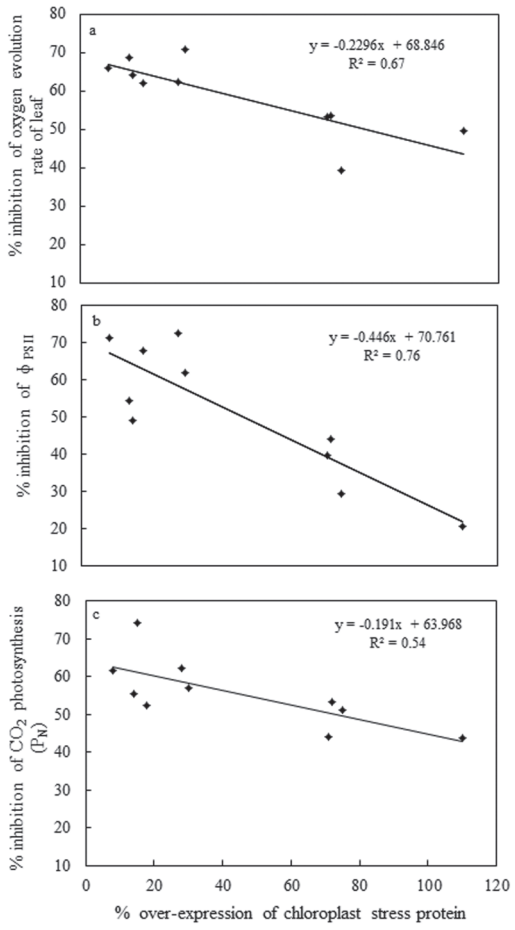

Fig. 6. Relationship between the 23 kDa stress protein abundance and percentage inhibition of (a) photosynthetic oxygen evolution rate, (b) $\Phi_{PS\,II}$ activity and (c) photosynthetic CO_2 assimilation rate

is generally considered to be drought susceptible. Earlier workers reported that this clone was relatively drought tolerant in traditional rubber growing areas (Rao *et al*., 1990). On the other hand, recent reports indicated that this clone is comparatively drought and high light susceptible in the traditional as well as non-traditional drought prone areas of India (Sreelatha *et al*., 2007; Annamalainathan *et al*., 2010; Thomas *et al*., 2011).

In an earlier study the 23.8 kDa stress protein was observed to be consistently over expressing in chloroplast thylakoid membrane of rubber plants experiencing drought and high solar light. A total of six different peptides from the induced stress protein successfully matched several sHSPs from tobacco, petunia and tomato (Annamalainathan *et al*., 2006). Stress proteins of similar kind were ascribed to associate with chloroplast functions related to photosynthetic activities, including PS II electron transport and oxygen evolution activity in the PS II (Torok *et al*., 2001; Barua *et al*., 2003; Heckathorn *et al*., 2004). Similar low molecular weight (LMW) stress proteins also protect photosynthetic electron transport from inhibitory effects of heavy metals (Kumar *et al*., 2015). Recently occurrence of a chloroplast HSP70B was correlated with oxidative stress response and defined as marker of oxidative stress in plants (Chankova and Yurina, 2016). Other than heat shock like proteins an array of regulatory and functional proteins like many transcription factors, proteins involved in signal transduction, LEA and other protein chaperons, proteases and ROS scavenging enzymes *etc.* are also induced under drought situations (Todaka *et al*., 2015).

Establishing young rubber plants in the field becomes difficult in the present scenario of climatic warming and frequent occurrences of drought. Drought situation is a common occurrence in recent past even in traditional rubber growing areas of many rubber growing countries (Satheesh and Jacob, 2011; Chantuma *et al*., 2012). Adverse environmental conditions limit the expansion of rubber cultivation to newer areas in several countries including India. In this context the present study which explains crucial photosynthetic measurements and analysis of stress protein abundance in the photosynthetic apparatus may be considered as one of the approaches towards developing screening tools (phenotyping) for identification of drought tolerant genotypes of natural rubber. However, it is a noteworthy to point that combining several markers may further improve the predictive value of right selections for environmental stress tolerance, high yield, disease tolerance *etc.* in *Hevea brasiliensis*.

CONCLUSION

A chloroplast membrane low molecular weight protein (23 kDa) was identified as a protein marker and its abundance was related to drought tolerance at the photosynthetic apparatus level. The relative abundance of this stress protein can be quantified using Western blot technique and employed as one of the screening tools along with analysis of critical physiological activities for the abiotic stress tolerance in young rubber plants.

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